Representative sampling and sample preparation in biological environmental monitoring using spruce shoots

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Received 5th October 2000, Accepted 8th March 2001
First published as an Advance Article on the web 4th April 2001

The design of sampling and sample processing has a tremendous effect on the analytical results from which conclusions are drawn with respect to the quality of the environment and any possible impact on human health. Large scale environmental surveys need rigorous planning and extensive screening experiments to evaluate their boundary conditions. Representativeness and sample homogeneity are highlighted here in the context of a long-term biological monitoring survey for trace elements using spruce shoots. Several aspects concerning the selection of samples, amount of material, material processing and sample preparation prior to chemical characterisation are illustrated by selected examples.

Introduction

The design of an environmental survey is clearly governed by both the objective and the capacity of the institutions involved. In environmental research not only is the assessment of quantities of hazardous substances in environmental matrices an important parameter but also the questions of mass balance, metabolic turnover, or accumulation in food webs should be addressed. Most of these problems can be investigated after detailed analysis of biological materials, as found in natural environments (active biomonitoring), or transplanted biomaterials (passive biomonitoring).

Plants and animals can integrate various properties of environmental quality into their tissues during their life cycle in a species specific manner.[1] The selection of a matrix from our natural surroundings for study is the first, albeit crucial, step in the necessary refinement to extract information from a principally heterogeneous real world system. Before this study material can be called representative for the extraction of meaningful analytical results related to the initial objective of the investigation it has to undergo various stages of refinement. Strategies for sampling have been discussed in the literature, mainly with regard to geological materials,[2–5] biomedical specimens[6,7] and for ecological investigations[8,9] but the collection of representative biological samples for environmental surveys has rarely been discussed.[10–12] The selection of species for a biomonitoring project should be concerned with aspects such as availability, accumulation characteristics and analytical requirements. Long-term projects additionally require consideration of concentration changes with age of the bioindicator or seasonal variation of their elemental composition.[13] The role and importance of biometric description (age, maturity stage, infection by pathogens, *etc.*) of the sampled specimen has been emphasised by several authors.[14–16] A rigid sampling protocol in the form of a standard operating procedure (SOP) has to be prepared if repeated sampling campaigns, in either time and/or location, are aimed at the extraction of time trends or comparison of different ecosystems with regard to pollution levels.[11,17]

The representativeness, in terms of chemical composition, of a matrix means that a small portion of the material should have comparable concentrations of the investigated analytes as compared with the whole population for which the investigation is carried out. The final analytical aliquot, which consists usually of a few hundred milligrams for elemental analysis (such as for metal determinations at the trace concentration level), should show mean concentrations as presented in the same matrix taken at random in the large study area (a defined ecosystem, a natural reserve area, a national park, *etc.*).

In addition to sample selection in the field, insufficiently homogenised aliquots will bias the analytical results strongly and lead to over- or underestimation of the environmental implications. This report intends to stress these points and to discuss some approaches to circumvent the constraints that might lead to erroneous conclusions in environmental monitoring surveys. Increased quality and reliability of monitoring data could result if some of the experiences from the Environmental Specimen Banking (ESB) projects[18,19] could be adopted.

The studies reported here have been performed in the framework of the further development of the Federal Environmental Specimen Bank of Germany, which was established in 1985 as a long-term environmental monitoring and banking project.[18] Spruce shoots are one example of the bioindicators that have been regularly sampled from various terrestrial ecosystems throughout Germany. The ESB samples have been chemically characterised with respect to a large number of analytes including trace metals. Over the years, several crucial aspects of the monitoring procedure outside the analytical laboratory, which can influence the information content of the ESB samples collected, have been identified and investigated as described in the following.

Sample selection and screening

The following aspects have to be considered for the design of an environmental biomonitoring study before starting the chemical analysis in the laboratory: (i) selection of an appropriate species by considering (a) the ecological role of the organism, (b) the function of the organism within the food web, (c) the accumulation properties, and (d) the influence of age, gender and/or the season; (ii) selection of a target organ by considering (a) is it easy to sample, (b) is there sufficient quantity, and (c) the analyte distribution; (iii) sample preparation by considering (a) the stability of the analyte, (b) milling and/or homogenisa-

 DOI: 10.1039/b008069l

tion, (c) aliquoting, *i.e.*, dividing the sample into small units suitable for various analytical investigations as well as storage of repository samples, and (d) contamination control.

This list emphasises only some of the important issues that need to be addressed prior to the final analytical operations, which include analytical sample preparation, *i.e.*, transfer of the analytes of interest into a measurable state (for instance, for metal analysis by digestion, dilution, *etc.*), and analyte determination. An example to demonstrate the importance of these aspects in environmental monitoring is given in Fig. 1. Errors in sampling strategy, such as an insufficient number of individual trees, selecting specific branches instead of random sampling, not having a well defined age class, contamination from sampling tools or containers, and insufficiently homogenised materials, will result in data but not in useful environmental information.

Therefore, before a large scale environmental survey is initiated, an investigation into the biological variability of an individual specimen should be carried out. As concentrations can vary significantly between individuals in a confined area, owing to genetic, micro-climatic or nutritional differences, representative sampling requires a statistically relevant number of randomly selected individuals (12–20 at least). In a screening experiment, the variability of analytes should be determined before a homogeneous, representative sample for the environmental survey can be obtained.

Experimental work

For almost 20 successive years under the ESB project, spruce shoots along with beech leaves, poplar leaves and pine shoots have been collected from different ecosystems as monitors for air pollution survey.[20] Spruce was chosen as it is a dominant tree in Germany and it is relatively well investigated with regard to its environmental response. The target organ is a one year old shoot in order to select a class with a defined exposure time. For instance, 30 individual randomly selected healthy trees were chosen from a forest ecosystem (Solling) in March

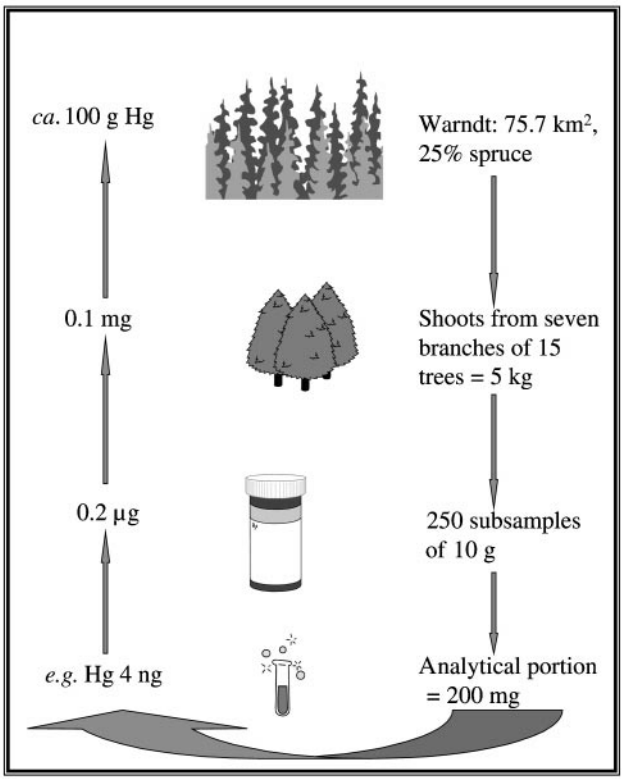

Fig. 1 Evaluation of Hg burden in spruce shoots of an ecosystem, Warndt, Saarland, Germany.

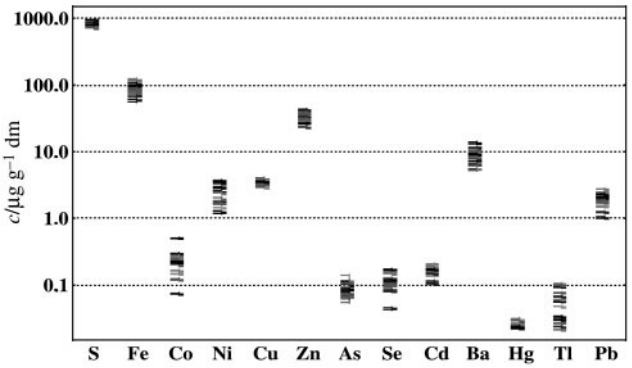

Fig. 2 Element concentrations of spruce shoots of 15 individual trees from Solling, Germany (dm = dry mass).

1999. Shoots from several branches of the seventh whirl counted from the top of each of these trees were collected and processed individually. Great care was taken to avoid external contamination (plastic gloves, stainless-steel tools, no contact with forest soil, *etc.*). Immediate deep-freezing and storage of the material at the sampling site in the vapour phase of liquid nitrogen ensured the absence of chemical degradation during transport. At the laboratory the material was cryogenically milled, using a large vibratory mill with titanium drum and rods, to yield a finely ground and fresh (including moisture) powder. Aliquoting and storage of the material in the vapour phase of liquid nitrogen guarantees the full integrity of the material without chemical changes to the constituents. After freeze-drying small parts of the homogenised sample (a few grams) the material was acid digested and analysed using a broad range of analytical methods for elemental analysis.[17,18,21,22] The spruce shoot samples were characterised regarding their content of S, Fe, Cu, Zn, Ba, Co, Ni, As, Se, Cd, Pb, Hg, Tl, and Cr using ICP-OES, ICP-MS, stripping voltammetry, HG-AAS, cold vapour-AAS, and isotope dilution-thermal ionisation MS. Regular quality assurance and control measures, such as blank checks, and analysis of certified (CRMs) and in-house reference materials, were an integral part of all the analytical procedures. Elements such as Cu, Cd, and Pb were analysed by at least two independent methods to avoid any analytical bias.

Fig. 2 shows the results from individual tree analysis using a logarithmic scale. As can be seen, the concentrations of certain elements (*e.g.*, Co, Ni, Se or Tl) vary by almost one order of magnitude whereas other elements (such as S, Cu or Hg) exhibit rather narrow bands of concentration. A statistical analysis of such results can help to estimate the minimum number of individuals that need to be sampled in order to obtain a representative material at a defined significance level. Based on the results of the screening, a sampling campaign for collection of the real survey sample can be designed. Random *versus* selected sampling, and sampling along transects or accidental selection of individuals (random generator) should be decided upon according to the objective of the study and the availability of the species. The larger the amount of material collected following such rules the lower will be the risk of bias and contamination. Within the ESB surveys, generally shoots from branches of the seventh whirl from 15 trees were sampled to make up about 5 kg of fresh material per field sampling.

The next crucial step in the process of preparing a meaningful sample for analysis is homogenisation of the material. As there are various approaches, with different pros and cons, some of these aspects will be discussed in more detail.

Homogenisation of samples

In order to obtain a material suitable for meaningful analysis, the crude sample, as collected from the environment, needs to

be thoroughly homogenised. This is an important step prior to analysis and much can be damaged if it is not carefully carried out. Experience has shown that simple drying and grinding of biological tissues with a pestle and mortar is generally insufficient to obtain a sample that properly reflects the natural concentrations. Most biological tissues consist of distinct subunits of different cell structures with different concentrations of constituents. It might be almost impossible to separate these entities entirely and so it is very difficult to homogenise the tissues properly, even if the most advanced milling devices are at hand.

Some materials, rich in either fibre or fat, have a tendency to smear or not to disintegrate into fine particles, as recommended for a representative analytical portion. In most of these cases, cryogenic milling offers the opportunity of solving such problematical homogenisation tasks. Using a ZrO_2 ball mill, or a Cryo-Palla® for larger amounts of material, the fresh and frozen materials can be cracked down to a fine particle size distribution. A further advantage of this procedure lies in the low temperature processing, which has a very low risk of loss of volatile constituents. Otherwise, owing to the sheering forces in the material, instantaneous overheating of up to 200 °C can happen in room temperature ball milling. In addition, the risk of contaminating the material increases with the amount of force and time used in the milling process. Therefore, several short milling intervals with low power are preferable to one long and forceful milling.

Finally, the result of the homogenisation process should be checked and, if necessary, the milling should be repeated until a suitable particle size distribution (80% < 100 μm) has been obtained. There are principally two aspects of checking the homogeneity of materials: the physical size, which can be measured using a laser particle sizer; and the chemical distribution, which can be described, *e.g.*, by relative homogeneity factors of single components in the materials.[23–25] The finer the material's particle size distribution the better should be the homogeneous distribution of components. But, unfortunately, this statement cannot be generalised and, hence, the actual component distribution must be checked individually for each analyte of interest. An example of a particle size distribution after cryogenic milling of a material is presented in Fig. 3.

This can provide only qualitative information on the distribution of analytes in the material. Each analyte exhibits an individual distribution pattern and should be checked individually. Repetitive analysis of small amounts of sample in a direct (no chemical dissolution) way is the preferred way of doing this.[26] Solid sampling-AAS (SS-AAS) is a convenient, fast and easy to handle technique for the accurate determination of a single element distribution for sample weights of 0.05–2.0 mg only. Instrumental neutron activation analysis (INAA) is more elaborate, needs specialised laboratories and access to a neutron source, but has multi-element capabilities and its

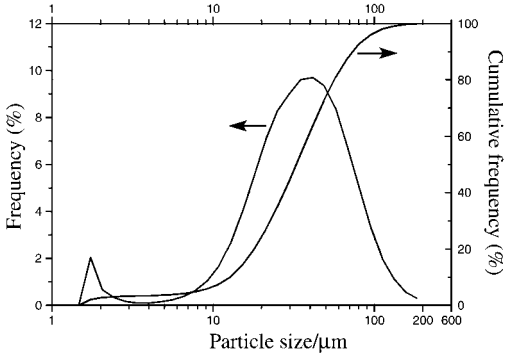

Fig. 3 Particle size distribution in lichen powder after cryogenic homogenisation.

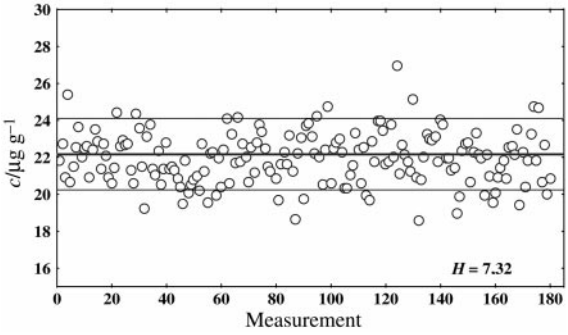

Fig. 4 Pb concentration in homogenised lichen (*Pseudevernia furfuralis*) from the Bavarian Forest, Germany, 1998. Mean sample mass: 0.821 ± 0.185 mg.

superb sensitivity allows the simultaneous analysis of 10–20 elements in biological materials on a sample weight of about 1 mg. Details of both techniques are given in refs. 25–27.

Repeated analyses of independent aliquots yields a mean value with a range of uncertainty, which is often expressed as the standard deviation. This reflects the reproducibility of the applied technique as well as the variability of the analyte, which is due to its distribution in the matrix. As the variances R from both contributions can be assumed to be additive, the variance for homogeneity (R_{ele}) can be derived after subtraction of the measurement contribution (R_{meas}) from the overall (R_{tot}) variance.

$$R_{tot}^2 = R_{meas}^2 + R_{ele}^2 \quad (1)$$

The relative homogeneity factor H_{rel} can be calculated according to eqn. (2):[28]

$$H_{rel} = \sqrt{R_{ele}^2 \cdot m} \quad (2)$$

where m is the mean sample mass used for the repeated analyses. The determination of element specific homogeneity factors opens the opportunity for a quantitative description of the analyte distribution in solids. This is particularly advantageous if direct methods are used for analysis of the materials under study or if the questionable reproducibility of the results from wet analysis techniques points towards badly homogenised materials. In several of these cases, by using SS-AAS, the hypothesis that inappropriate materials might be the cause of the variability in the results might not be proved and other reasons within the analytical process would have to be identified.

Examples for the extraction of element specific homogeneity factors from measurements obtained with SS-AAS are given in Figs. 4, 5 and 6. In Fig. 4 individual measurement results are displayed from a homogeneity study of Pb in homogenised lichen material. From Figs. 5 and 6 it can be seen that the instrumental uncertainty of the results using liquid (perfectly

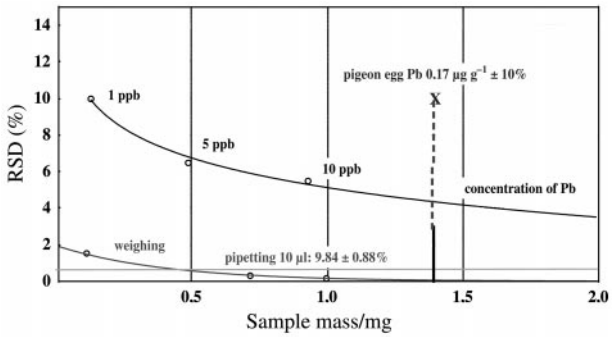

Fig. 5 Uncertainty contribution from pipetting, weighing and concentration related variability in SS-AAS for Pb determination.

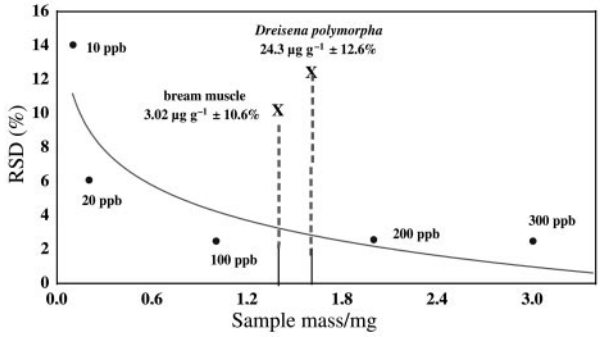

Fig. 6 Uncertainty contribution from concentration related variability in SS-AAS for Cu determination.

homogeneous) samples declines with increasing concentration of the solution (upper solid line). For Pb, we assume an asymptotic behavior for concentrations in excess of 100 ng g^{-1} resulting in a RSD limit of around 3%. For Cu, a conservative estimate provides a limiting RSD of 2% for the concentrations found in biological tissues. This instrumental contribution has to be subtracted according to eqn. (1) from the total uncertainty, which has been determined by repeated SS-AAS analyses (120–160 measurements). The resulting uncertainty is then related to the elemental contribution resulting from the heterogeneous distribution. In Figs. 5 and 6 the X signifies the measured uncertainty from SS-AAS experiments at a sample intake of 1.45 mg for pigeon eggs, 0.26 mg for bream muscles and 0.135 mg for zebra mussel tissue (*Dreissena polymorpha*).

In Table 1 are summarised the H_{rel} values in different matrices for the elements investigated so far. The table clearly demonstrates the material- and element-specific characteristics of analyte distributions. Cd was found to be generally more homogeneous (lower H_{rel} values) than Pb, but Cu distribution can be distinctly different in the same matrix from different places (*e.g.*, bream muscle from Blankenese and Barby, or Cr in roe deer liver from Warndt, Dübener Heide, Bornhöved and Berchtesgaden). Although the materials have been processed according to SOPs[17] under cryogenic conditions with the same milling device, resulting in similar particle size distributions for each matrix, the chemical distribution of the elements in the homogenate might be distinctly different.

As the distribution of analytes in a certain matrix has (in dependence of the sample mass consumed for analysis) a crucial influence on the analytical repeatability as well as on the mean result of a limited number of repetitions, it is clear that any representative material used for environmental investigations needs to be carefully checked for homogeneity, and that such data should be documented before the results are interpreted in an eco-systematic context. What has been shown for chemical elements still needs to be demonstrated for organometallic species and organic constituents (*e.g.*, methyl mercury, chlorinated hydrocarbons, PAHs).

Conclusion

Interpretation of environmental data from biomonitoring programmes requires careful evaluation of all the steps from selection to preparation of the sample in order to ensure that the analytical results obtained truly represent the aim of the investigation. In the process of sample reduction from that in the ecosystem towards the small analytical portion used for the determination many mistakes can be made, thereby hampering the final extrapolation of the results to the interpretation of the environmental status in the study area. Proof of representative sampling, together with the quality assurance measures for sampling and sample processing (such as parallel sampling by independent teams of experts, strict adherance to SOPs for the whole process, minimising the number of operations for sample manipulation, regularly checking for potential contamination sources, *etc.*) are still difficult steps within the process of monitoring. Demonstration of a homogeneous analyte distribution in the study matrix can be achieved quantitatively by replicate SS-AAS or INAA measurements at small sample mass levels and the determination of H_{rel} values. The reliability of environmentally related research results will be enhanced when QA/QC measures concerning the representativeness of the samples investigated are strictly observed.

Table 1 Relative homogeneity factors H_{rel} (% $mg^{0.5}$) for Cr, Ni, Cu, Cd, and Pb in different environmental matrices

Matrix	Origin	Cr	Ni	Cu	Cd	Pb
Eelpout muscle *Zoarces viviparus*	Darßer Ort			4.64		
	Jadebusen			11.41		
Brown algae *Fucus vesiculosus*	Eckwarderhörne				4.77	
	Königshafen				5.69	
	Varnkevitz				5.19	
Bream muscle *Abramis brama*	Blankenese			5.44		
	Barby			29.9		
Bream liver *Abramis brama*	Bornhöved		186.2			
	Blankenese		114.3			
Beech leaves *Fagus sylvatica*	Bayerischer Wald	21.73				7.31
Zebra mussel *Dreissena polymorpha*	Rehlingen			4.63	1.99	
	Weil/Rhein				3.79	
Pine shoots *Pinus sylvestris*	Dübener Heide					11.2; 39.5
Common mussel *Mytilus edulis*	Eckwarderhörne				7.19	
	Darßer Ort				3.3	
Poplar leaves *Populus nigra Italica*	Leipzig				7.86	13.84
	Saartal	21.01				
Earth worm, defecated *Lumbricus terrestris*	Saartal			5.11		
	Bornhöved			3.22		
Roe deer liver *Capreolus capreolus*	Warndt	7.5; 24.9				30.8
	Dübener Heide	5.9				24.3
	Bornhöved	30.9				
	Berchtesgaden	14.5				
Herring gull egg *Larus argentatus*	Trischen			4.15		
	Heuwiese			7.09		
Pigeon egg *Columba livia f. dom*	Saartal			1.72; 2.63		12.0
Lichen *Pseudevernia furfuralis*	Bayerischer Wald	7.74				6.6

Acknowledgements

The team of the Environmental Specimen Bank at the Research Centre Jülich as well as the people involved at the cooperating Institutes at the University of the Saarland, the GSF Neuherberg, the Biochemisches Institut für Umweltcarcinogene, Großhansdorf, and many other reseach fellows have helped to accumulate experience in sampling, sample preparation and analysis over the last two decades. All of them are gratefully acknowledged and deserve our full respect.

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